Virology **292,** 285–298 (2002)
doi:10.1006/viro.2001.1236, available online at http://www.idealibrary.com on IDEAL®

Vaccinia Virus CrmE Encodes a Soluble and Cell Surface Tumor Necrosis Factor Receptor That Contributes to Virus Virulence[1]

Patrick C. Reading, Anu Khanna,[2] and Geoffrey L. Smith[3]

Department of Infectious Diseases, Division of Investigative Science, Faculty of Medicine, Imperial College, St. Mary's Campus, Norfolk Place, London W2 1PG, United Kingdom

Received August 31, 2001; returned to author for revision October 2, 2001; accepted October 23, 2001

Poxviruses encode soluble cytokine receptors to interfere with host immune functions. Cells infected with vaccinia virus (VV) strains USSR, Lister, and Evans express soluble and cell surface tumor necrosis factor receptors (vTNFRs). We have characterized vTNFR activity in VV USSR and identified an open reading frame that encodes both soluble and cell surface activity, hereafter referred to as VV cytokine response modifier E (VV CrmE). Expression and characterization from recombinant VV and baculovirus showed VV CrmE to be an 18-kDa protein that bound human, mouse, and rat TNF-α, but not human LTα. VV CrmE inhibited the cytotoxic and apoptotic activities of human, but not mouse or rat, TNF *in vitro.* Nonetheless, in a murine intranasal model, USSR recombinants lacking CrmE were attenuated, demonstrating a role *in vivo.* Furthermore, expression of VV or cowpox virus vTNFRs from VV strain WR (which itself does not express a vTNFR) was shown to enhance virulence in the murine model.

Key Words: vaccinia virus; cowpox virus; tumor necrosis factor; virulence; cell surface; apoptosis; necrosis; lymphotoxin α.

INTRODUCTION

Poxviruses are large DNA viruses that encode many proteins that interfere with host immune functions, including several that function as soluble cytokine receptors or binding proteins. Poxvirus proteins that bind tumor necrosis factor (TNF), interleukin 1β (IL-1β), IL-18, interferon-γ (IFN-γ), IFN-α/β, and CC chemokines have been described; for reviews see Alcamí and Koszinowski (2000) and Smith (2000).

The family of TNF-like molecules comprises an increasing number of secreted and membrane-bound proteins, including TNFα and lymphotoxin α (LTα), reviewed by Locksley *et al.* (2001). Soluble TNF and LTα function as trimers, binding to two distinct receptors, TNFR1 (p55) and TNFR2 (p75), to induce receptor oligomerization and subsequent activation of intracellular signaling cascades. Members of the TNFR family exhibit a characteristic extracellular region consisting of a repeating cysteine-rich domain (CRD) that mediates ligand binding; however, the intracellular domains involved in signal transduction are more diverse. TNFR1 and TNFR2 are expressed on virtually all cells of the body, with the exception of red blood cells, and binding of TNFs to receptors can induce a variety of responses including cellular activation, differentiation, or death.

TNF is a pleotropic cytokine associated with many cellular functions, including inflammation and antiviral defense. TNFs and TNFRs mediate antiviral activity in a number of ways, including selective killing of virus-infected cells, inhibition of virus replication, and the induction of an antiviral state in uninfected cells (Mestan *et al.,* 1986; Wong and Goeddel, 1986; Wong *et al.,* 1988). TNFs are induced in response to a range of viral infections and often act synergistically with IFN-γ to mediate antiviral responses (Wong *et al.,* 1988; Lucin *et al.,* 1994). Furthermore, TNF is a proinflammatory cytokine involved in the recruitment of immune cells to sites of infection, thereby aiding clearance of virus. *In vivo,* a recombinant vaccinia virus (rVV) expressing TNFα was highly attenuated in a mouse model of infection (Sambhi *et al.,* 1991).

The physiological significance of TNF in poxvirus infections is underscored by the observation that these viruses encode proteins which interfere with intracellular components downstream of TNF–TNFR binding or which are secreted to bind and sequester TNF. Serine protease inhibitors (serpins) encoded by cowpox, rabbitpox, variola, and vaccinia viruses inhibit intracellular caspases, thereby mediating a number of different functions including the blockade of TNF-mediated apoptotic cell death (Dobbelstein and Shenk, 1996; Kettle *et al.,* 1997). Genes encoding proteins with sequence similarity to the N-terminal ligand-binding CRDs of cellular TNFRs have also been identified in several poxviruses. The first pox-

[1] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. (USSR B28R/C22L) AJ416892, (A53R) AJ416893, and (CrmE) AJ315962.

[2] Present address: Warden's Flat 4, Bramaputra Hostel, Poorvanchal, Jawaharlal Nehru University, New Delhi 110067, India.

[3] To whom correspondence and reprint requests should be addressed at Department of Infectious Diseases, Room 333, Division of Investigative Science, Faculty of Medicine, Imperial College, St Mary's Campus, Norfolk Place, London W2 1PG, UK. Fax: +44-207-594-3973. E-mail: glsmith@ic.ac.uk.

0042-6822/02 $35.00

virus TNFR (vTNFR) characterized was from Shope fibroma virus (SFV) where expression of the SFV T2 open reading frame (ORF) produced a soluble glycoprotein that bound human TNF and LT (Smith *et al.,* 1990). A similar protein was identified in myxoma virus, and deletion of the vTNFR gene (M-T2) attenuated the virus in a rabbit model of infection (Upton *et al.,* 1991). Interestingly, multiple different vTNFRs have been detected in some orthopoxviruses. Cowpox virus (CPV) strain Brighton Red encodes three different vTNFRs, termed cytokine response modifier B (CrmB; 48 kDa) (Hu *et al.,* 1994), CrmC (25 kDa) (Smith *et al.,* 1996), and CrmD (46 kDa) (Loparev *et al.,* 1998), that vary with respect to ligand specificity (CrmB and CrmD bind human TNF and LTα, and CrmC binds human TNF but not LTα) and time of expression (CrmB is expressed early, and CrmC and CrmD are expressed late). All three vTNFRs contain an N-terminal domain that shares amino acid similarity with the four CRDs of host TNFRs; however, CrmB and CrmD encode a unique ~160-amino-acid C-terminal domain of unknown function. Sequence analysis of CPV GRI-90 identified a fourth ORF (K3R) predicted to encode a protein of 167 amino acids with sequence similarity to members of the TNFR superfamily (Shchelkunov *et al.,* 1998) and the related gene in CPV strain elephantpox virus (EP) was shown to encode a soluble vTNFR, designated CrmE (Saraiva and Alcamí, 2001). A CrmB-like protein is the only functional vTNFR predicted from known variola and camelpox virus sequences (Aguado *et al.,* 1992; Massung *et al.,* 1994; Shchelkunov *et al.,* 2000; Saraiva and Alcamí, 2001), while an intact CrmD is predicted in the Moscow strain of ectromelia virus (Loparev *et al.,* 1998).

VV strains Western Reserve (WR) and Copenhagen encode two vTNFR genes, A53R and C22L/B28R (present within the inverted terminal repeat and therefore diploid), that correspond to cowpox CrmC and CrmB, respectively. These VV vTNFR genes are truncated due to frameshift or nonsense mutations and were predicted to be inactive (Goebel *et al.,* 1990; Howard *et al.,* 1991; Upton *et al.,* 1991). Although most VV strains do not produce vTNFRs, VV strains Lister, USSR, and Evans encode soluble vTNFR activity (Alcamí *et al.,* 1999). In VV strain Lister, the A53R gene encodes an active soluble vTNFR, while gene B28R is truncated by mutations. Interestingly, vTNFRs are also expressed at the surface of cells infected with VV strains USSR, Lister, and Evans but not by CPV or camelpox virus, and the gene(s) responsible for this activity is unknown.

Here we have characterized soluble and cell surface vTNFR activity encoded by VV USSR. We found that the USSR A53R gene encoded soluble vTNFR activity and identified an ORF in VV USSR that encoded a vTNFR similar to the CrmE of CPV EP. VV CrmE was characterized further following expression from baculovirus and recombinant VV WR and found to mediate soluble and membrane-bound TNF-binding activity in both systems. Finally, USSR deletion mutants lacking VV CrmE, but not CrmC, were attenuated in a murine intranasal model, demonstrating a role for this vTNFR *in vivo.*

RESULTS

Analysis of A53R and B28R regions in VV strain USSR

In VV Lister, gene A53R encodes a soluble vTNFR but gene B28R is truncated by mutations that introduce stop codons or frameshifts (Alcamí *et al.,* 1999). The sequence of genes A53R and B28R was determined in VV USSR because this virus is more virulent in mice than VV Lister (A. Khanna, A. Alcamí, and G. L. Smith, unpublished results) and would be more appropriate for experiments to determine the role of vTNFRs in virus virulence. The A53R and B28R genes were cloned from VV USSR as described under Materials and Methods. The A53R ORF of USSR encoded a predicted polypeptide of 186 amino acids with an N-terminal hydrophobic sequence and two potential N-linked glycosylation sites. VV USSR A53R has 98% amino acid identity to VV Lister A53R and showed sequence similarity to members of the TNFR superfamily. The VV USSR B28R gene was found not to encode an intact vTNFR, but only short ORFs that, when assembled together by introduction of frameshifts, produced a complete vTNFR. The USSR B28R DNA sequence shared 98% nucleotide identity with the Lister B28R gene (Alcamí *et al.,* 1999) and all nonsense and frameshift mutations were conserved.

Deletion of A53R gene in VV strain USSR

The A53R and B28R gene sequences in VV USSR suggested that only A53R encodes a functional vTNFR. To study the function of the A53R-encoded vTNFR, approximately 88% of this gene was deleted from VV USSR by transient dominant selection (vΔA53R). A revertant virus was also constructed by reinserting the A53R ORF into the deletion virus (vA53R-rev). Analysis of the genomes of vΔA53R, wild-type (vWTA53R), and vA53R-rev viruses by PCR confirmed that most of the A53R gene was deleted from vΔA53R and that the genes flanking the A53R gene were of the expected size. Thus, the genomic structures of the viruses around the A53R locus were as predicted. Furthermore, the plaques formed on BS-C-1 cells by vWTA53R, vΔA53R, and vA53R-rev viruses were the same size, and the yield of intracellular virus produced at 24 h postinfection (p.i.) at 1 PFU/cell in BS-C-1 cells was indistinguishable for each virus (data not shown), indicating that the A53R gene is nonessential for replication *in vitro.*

Thymidine kinase-negative (TK^-)143B cells were infected with rVVs and at 24 h p.i. the secreted and cell surface vTNFR activity was determined in binding assays with ^{125}I-labeled human TNF. Despite deletion of A53R, vΔA53R still expressed soluble and membrane-bound vTNFR to levels comparable to those of vWTA53R and

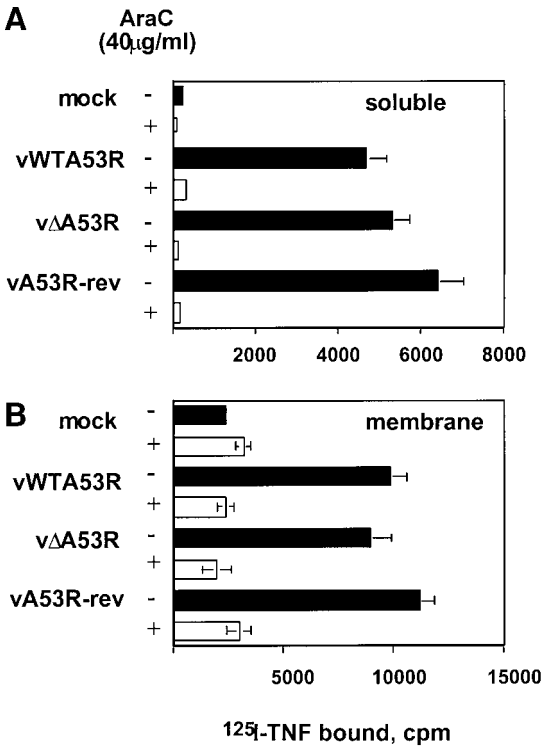

FIG. 1. TNF-binding activity of vΔA53R USSR. (A) Soluble binding assay. Supernatants from cell cultures infected with the indicated viruses, in the absence (−) or in the presence (+) of AraC, were incubated with human ^{125}I-TNF. Bound ^{125}I-TNF was determined by precipitation with PEG and filtration. Specific ^{125}I-TNF binding of duplicate samples (mean ± SEM) after subtraction of background radioactivity is shown. (B) Membrane-binding assay. Cells infected with the indicated viruses, in the absence (−) or in the presence (+) of AraC, were detached from the plate, washed in binding medium, and incubated for 2 h at 4°C with human ^{125}I-TNF. Bound ^{125}I-TNF was determined after phthlate oil centrifugation by measuring radioactivity associated with the cell pellet in a gamma counter.

vA53R-rev viruses (Fig. 1). This suggested the existence of an additional vTNFR gene(s) in VV USSR and suggested that this gene(s) encodes the majority of both soluble and cell surface TNF-binding activity in VV USSR. No vTNFR activity (membrane or soluble) was found when cells were infected with vΔA53R in the presence of cytosine arabinoside (AraC), an inhibitor of DNA synthesis and therefore of intermediate and late viral gene expression. AraC also inhibited vTNFR expression by vWTA53R and vA53R-rev (Fig. 1), confirming that both A53R and the gene(s) encoding the additional vTNFR/s were expressed late during infection.

Identification of an additional ORF in VV strains USSR, Lister, and Evans that encodes a soluble and cell surface vTNFR

Recently, a putative protein (called K3R) with sequence similarity to the TNFR superfamily was identified in CPV strain GRI-90 (Shchelkunov *et al.,* 1998). The protein encoded by this ORF, designated CrmE, was characterized in CPV strain EP and found to encode a soluble 18-kDa vTNFR that bound human, mouse, and rat TNF-α (Saraiva and Alcami, 2001). Combinations of specific oligonucleotide primers designed to GRI-90 K3R amplified the K3R counterpart from genomic DNA of VV USSR. Cognates in other VV genomes were identified by PCR and Southern blot hybridizations. The gene was identified and sequenced in VV strains USSR, Lister, and Evans, but could not be detected in WR, Copenhagen, IHD-J, IHD-W, Wyeth, Lister, Tian-Tan, Tashkent, King Institute, Dairen, Patwadangar, rabbitpox, or buffalopox. The deduced amino acid sequence of CrmE from VV USSR, Lister, and Evans was identical and predicted an 18-kDa polypeptide with a putative 17-amino-acid N-terminal signal peptide and a single potential N-linked glycosylation site at residue 71. VV CrmE differed by a single amino acid substitution from CPV strain GRI-90 K3R (L → P at residue 42) and CPV strain EP CrmE (S → P at residue 82).

The presence of a 17-amino-acid N-terminal signal peptide in VV CrmE suggested that the protein could be secreted. Furthermore, it was reported that EP and a rVV WR expressing EP CrmE produced soluble but not cell surface vTNFR activity (Saraiva and Alcamí, 2001). To address if the VV CrmE encoded soluble and/or cell surface vTNFRs, approximately 85% of the ORF was deleted from VV USSR (vΔCrmE) and vΔA53R (vΔA53RΔCrmE) by transient dominant selection. Both wild-type and deletion mutant viruses were produced from the same intermediate mycophenolic acid (MPA)-resistant parent viruses (vWTCrmE and vWTΔA53R, respectively), and revertant viruses were produced by reinsertion of CrmE into the deletion viruses (vCrmE-rev and vΔA53RCrmE-rev, respectively). PCR analyses confirmed the genomic structures of all viruses around both the A53R and the CrmE loci (data not shown), and no differences in plaque phenotype or yield of intracellular virus were noted following infection of BS-C-1 cells with these viruses (data not shown), indicating that CrmE is nonessential for replication of VV USSR *in vitro.*

Cells infected with deletion mutants lacking the CrmE counterpart (i.e., vΔCrmE and vΔA53RΔCrmE) displayed low levels of either membrane or soluble vTNFR activity (Fig. 2). Significant vTNFR activity was detected in supernatants from cells infected with vΔCrmE, consistent with the presence of an intact A53R; soluble vTNFR activity was reduced further in supernatants from cells infected with vΔA53RΔCrmE. Together, these results indicate that CrmE is responsible for the majority of both soluble and cell surface vTNFR activity expressed by VV USSR. Our results also indicate that CrmE is more abundant in supernatants than at the cell surface; 1.5×10^6 cells infected with vWTΔA53R (expressing CrmE but not A53R) bound approximately 8000 cpm of ^{125}I-TNF at the cell surface (Fig. 2B), compared to 5500 cpm bound by medium from 1.5×10^5 infected cells (Fig. 2A).

Previous studies have shown that supernatants from VV Lister- or CPV-infected cultures do not confer vTNFR

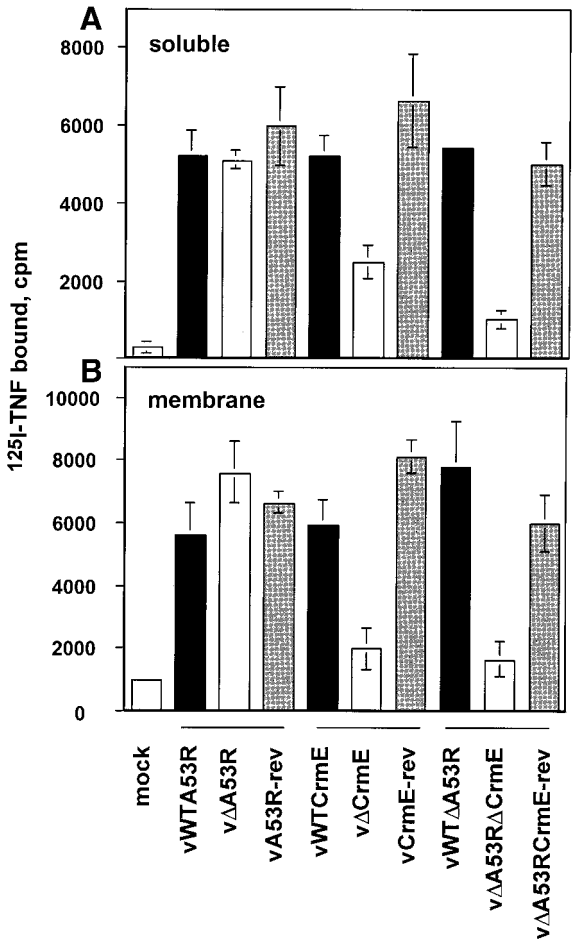

FIG. 2. VV USSR CrmE encodes both soluble and cell surface vTNFR activity. (A) Soluble vTNFR activity produced by USSR-derived recombinant viruses. Monolayers of TK⁻143B cells were mock-infected or infected with the indicated viruses at 10 PFU/cell, and the supernatants were harvested 24 h p.i. Specific ^{125}I-TNF binding of duplicate samples (mean ± SEM) after subtraction of background with medium is shown. (B) Membrane vTNFR activity in USSR-derived recombinant viruses. TK⁻143B cells were mock-infected or infected with the indicated viruses and tested for cell surface TNF-binding activity at 24 h p.i. Bound human ^{125}I-TNF was determined after phthlate oil centrifugation by measuring radioactivity associated with the cell pellet in a gamma counter.

activity at the surface of mock- or VV WR-infected cells (Alcamí *et al.,* 1999). To confirm that cell surface TNFR activity of VV USSR was not due to attachment of the secreted receptor to the surface of virus-infected cells, perhaps due to interaction with an additional protein encoded by VV USSR, mock-infected or vΔCrmE-infected cells were preincubated with supernatants from cultures infected with WTA53R, vΔA53R, or vΔCrmE which contained soluble vTNFR activity; no enhancement of cell surface vTNFR activity was noted in mock- or vΔCrmE-infected cultures (data not shown).

Expression of VV crmE from VV WR and baculovirus

USSR CrmE was expressed from VV WR (which does not express vTNFR activity) and from recombinant baculoviruses under the control of strong promoters. A protein of 18 kDa was detected in cell pellets and supernatants from (i) TK⁻143B cells infected with a rVV expressing USSR CrmE (vCrmEUSSR) and (ii) Sf21 (*Spodoptera frugiperda* 21) cells infected with a recombinant baculovirus expressing USSR CrmE (AcCrmEUSSR), but was absent from cultures infected with wild-type VV WR or AcNPV (Fig. 3A). A similar protein was also secreted into supernatants of cells infected with rVVs or recombinant baculoviruses expressing CrmE from VV Lister and Evans (data not shown). As reported previously (Alcamí *et al.,* 1999), cells infected with recombinant VV or baculoviruses expressing A53R from VV Lister (vA53RLister and AcA53RLister, respectively) secreted a protein of approximately 20 kDa (Fig. 3A).

To determine if the VV CrmE-like protein is modified by glycosylation, cells were infected with vCrmEUSSR in the presence or absence of either tunicamycin (an inhibitor of N-linked glycosylation) or monensin (which alters protein translocation through the Golgi network, thereby inhibiting addition of O-linked glycosylation). While tunicamycin had no effect upon the size or the secretion of CrmE from vCrmEUSSR-infected cells, secretion was inhibited in the presence of monensin (data not shown). The ability of monensin to inhibit CrmE secretion may indicate a direct effect on the addition of O-linked carbohydrate to the protein or perhaps an indirect effect upon the processing of the protein within the Golgi network. As a control, another VV-encoded protein, A41L, had a decreased size but was still secreted from infected cells in the presence of either tunicamycin or monensin, indicating the presence of both N- and O-linked carbohydrate (data not shown) (Ng *et al.,* 2001).

Deletion of A53R from VV USSR has little effect upon levels of soluble or cell surface vTNFR activity (Fig. 1); however, deletion mutants lacking CrmE produce very little of either vTNFR activity (Fig. 2). Therefore it was of interest to determine the soluble and cell surface TNF-binding activity from cells infected with rVVs and baculoviruses expressing VV CrmE or A53R. TK⁻143B cells infected with vCrmEUSSR produced both secreted and cell surface vTNFRs, while vA53RLister-infected cells produced only soluble TNF-binding activity (Fig. 3B). Similar results were obtained after infection of BS-C-1, RK_{13}, or CV-1 cells (data not shown), demonstrating that the expression of cell surface vTNFR activity is not particular to TK⁻143B cells. Cell surface vTNFR activity was also detected in Sf21 insect cells infected with AcCrmEUSSR (Fig. 3B), indicating that CrmE need not be expressed in the context of VV infection to mediate cell surface vTNFR activity. Again, soluble, but not cell surface, vTNFR activity was noted following infection with baculovirus expressing Lister A53R (AcA53RLister).

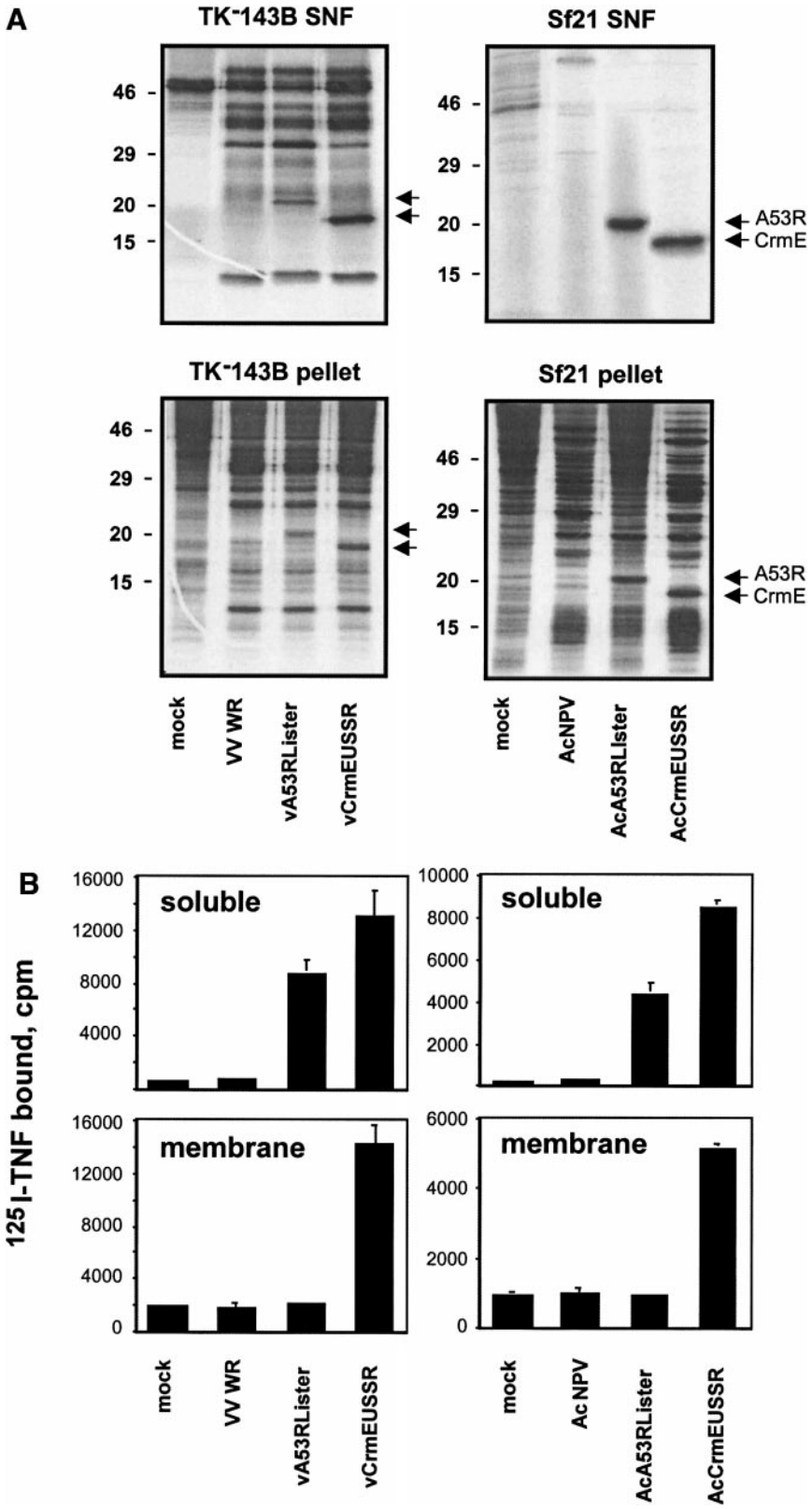

FIG. 3. Expression of vTNFRs from VV USSR by recombinant VVs and by recombinant baculoviruses. (A) Metabolic labeling of proteins from cultures infected with recombinant viruses. TK$^-$143B cells were mock-infected or infected with the indicated orthopoxviruses and pulse-labeled with [^{35}S]cysteine and [^{35}S]methionine from 4 to 8 h p.i. Sf21 cells were mock-infected or infected with the indicated recombinant baculoviruses and pulse-labeled from 24 to 26 h p.i. Proteins present in cell extracts and supernatants (SNF) were analyzed by SDS–PAGE and fluorography. The positions of USSR vTNFRs A53R and CrmE are indicated. Molecular masses in kilodaltons are shown. (B) TNF-binding activity from cultures infected with recombinant viruses. TK$^-$143B or Sf21 cells were mock-infected or infected with VVs or baculoviruses, respectively. At 24 h p.i. supernatants were harvested and 100 μl was tested for binding of ^{125}I-labeled human TNF in a soluble binding assay. Specific ^{125}I-TNF binding of duplicate samples (mean $\pm$ SEM) after subtraction of background with medium is shown. Mock-infected and infected cells were also tested for cell-surface TNF-binding activity. Bound ^{125}I-TNF of duplicate samples (mean $\pm$ SEM) was determined after phthlate oil centrifugation by counting radioactivity associated with the cell pellet in a gamma counter.

Binding properties of VV USSR CrmE

The specificity of the CrmE-like vTNFR was analyzed in binding assays with culture supernatants or cells from vCrmE-USSR-infected cells and human ^{125}I-TNF in the presence of excess unlabeled human LTα or TNF from human, mouse, and rat (Fig. 4). Addition of a 1000-fold excess of TNF from human, mouse, or rat reduced soluble vTNFR activity by 90–95% (Fig. 4A); however, there was no effect on TNF binding by the vTNFR in the presence of an excess of unlabeled human LTα. Similar results were obtained with culture supernatants from TK$^-$143B cells infected with vΔA53R or from Sf21 cells infected with AcCrmEUSSR (data not shown). Similarly, the membrane-bound vTNFR activity in vCrmEUSSR-infected cells was reduced by 80–90% in the presence of a 1000-fold excess of TNF from human, rat, or mouse (Fig. 4B). A 1000-fold excess of cold LTα had no effect on the cell surface vTNFR, indicating a very low affinity for this ligand.

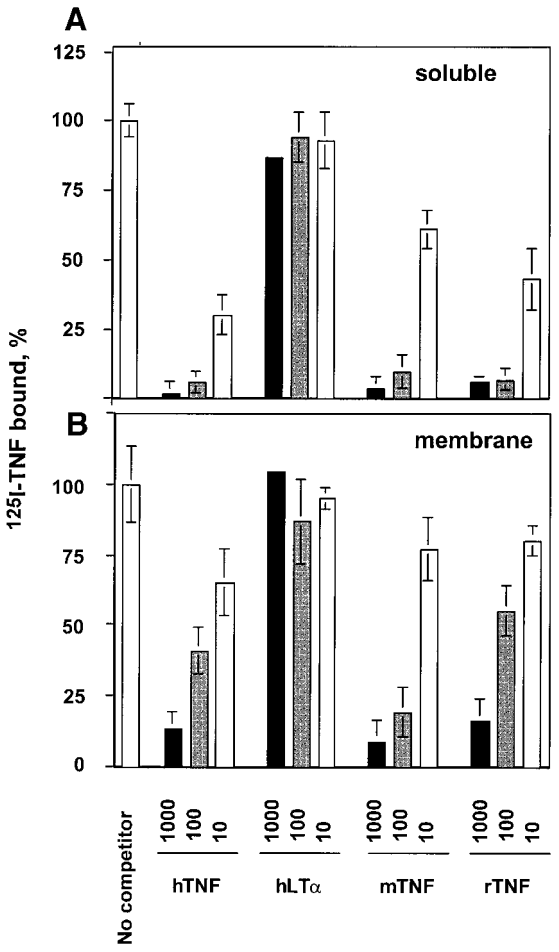

FIG. 4. Binding specificity of VV CrmE. (A) Soluble binding assay. Supernatants from cell cultures infected with rVVs expressing USSR CrmE (vCrmEUSSR, equivalent to 2×10^4 cells) were incubated with human ^{125}I-TNF in the presence or in the absence of the indicated fold excess of unlabeled human TNF (hTNF), human LTα (hLTα), mouse TNF (mTNF), or rat TNF (rTNF). Bound ^{125}I-TNF was determined after precipitation with PEG and filtration. The percentage of ^{125}I-TNF binding of duplicate samples (mean ± SEM) refers to binding in the absence of competitor. (B) Membrane-binding assay. Cells infected with vCrmEUSSR were detached from the plate, washed with binding medium, and incubated for 2 h at 4°C with human ^{125}I-TNF in the absence or in the presence of the indicated fold excess of unlabeled hTNF, LTα, mTNF, or rTNF. Bound ^{125}I-TNF was determined after phthlate oil centrifugation. Results are expressed as the percentage binding relative to that recorded in the absence of competitor.

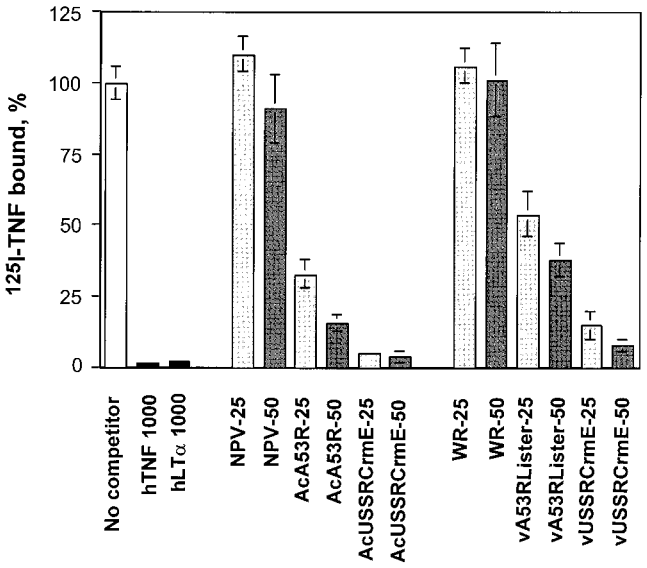

FIG. 5. VV vTNFRs inhibit binding of ^{125}I-TNF to U937 cellular receptors. Supernatants from Sf21 cells infected with the indicated baculoviruses or TK$^-$143B cells infected with VVs were incubated with 200 pM human ^{125}I-TNF for 2 h on ice, and the amount of cell-associated ^{125}I-TNF was determined following phthlate oil centrifugation. The amount of medium used was 25 or 50 μl and corresponded to $\sim 4 \times 10^4$ or $\sim 8 \times 10^4$ cells, respectively. Binding was also performed in the presence of a 1000-fold excess of unlabeled hTNF or LTα. Data are expressed as a percentage of counts bound to cells in the presence of competitor relative to that without (mean ± SEM).

Next we investigated the ability of VV vTNFRs to block binding of human TNF to cellular receptors. Supernatants from TK$^-$143B cells infected with vCrmEUSSR inhibited binding of human ^{125}I-TNF to U937 cells more efficiently than did supernatants from vA53RLister-infected cells (Fig. 5). This difference was also observed using supernatants from AcCrmEUSSR and AcA53RLister-infected insect cells or mammalian cells infected with vΔA53R and vΔCrmE (data not shown).

Biological activity of the soluble VV USSR CrmE

TNF-induced cell death in L929 cells is characterized by a necrosis-like phenotype and is dependent on the production of mitochondrial reactive oxygen species (Goossens *et al.,* 1995). To examine inhibition of biological activity of TNF by soluble VV vTNFRs, cytotoxic assays were performed using murine L929 cell targets exposed to human, murine, or rat TNF or to human LTα. Supernatants from vCrmEUSSR-infected cells were a potent inhibitor of the lytic activity of human TNF, but not murine or rat TNF, against L929 cells (Fig. 6). In contrast,

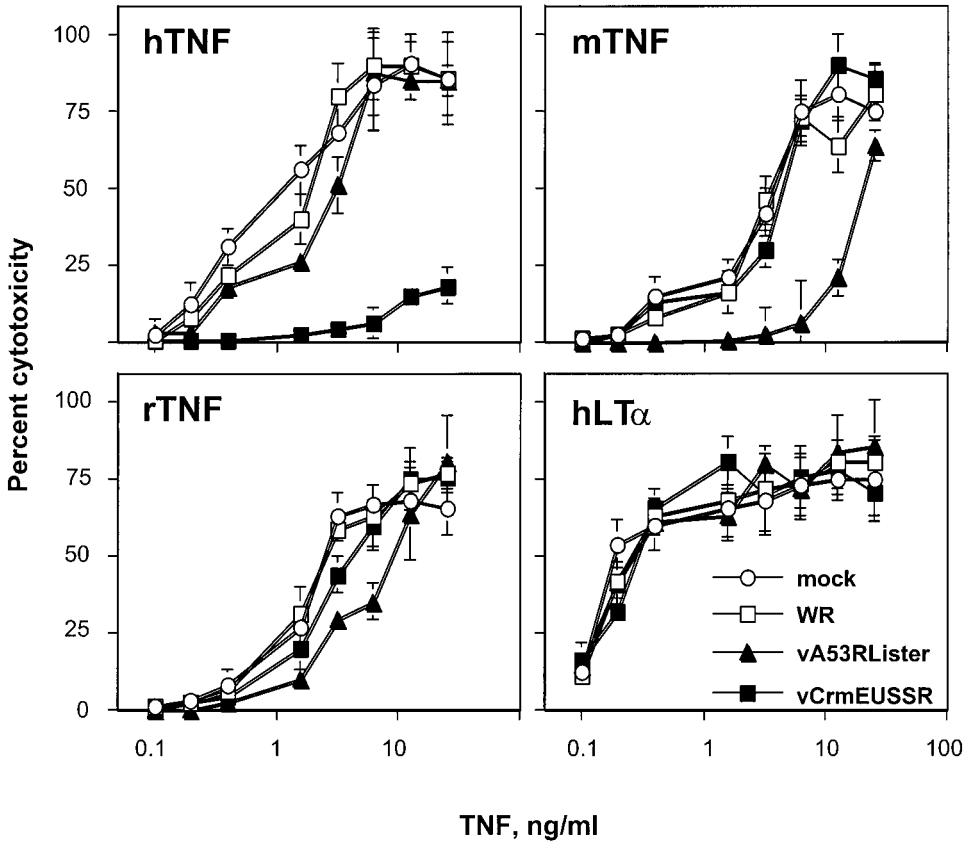

FIG. 6. Inhibition of TNF-mediated cellular cytotoxicity by VV vTNFRs. Crystal violet staining was used to determine the percentage cell viability after 12 h of treatment with hTNF, mTNF, rTNF, or LTα as indicated. Triplicate dilutions of TNFs were incubated for 1 h at 4°C with 20 μl of supernatant from TK$^-$143B cells that had been mock-infected (○) or infected with WR (□), vA53RLister (▲), or vCrmEUSSR (■), prior to their addition to L929 cells. Results are presented as percentage cytotoxicity relative to an OD control in the absence of TNF.

vA53RLister inhibited murine and, to a lesser extent, rat TNF-mediated cytolysis but had little effect on human TNF. Neither USSR CrmE nor Lister A53R was found to block the cytolytic activity of human LTα.

The finding that CrmE inhibits the cytotoxic activity of human TNF only is somewhat surprising since murine TNF and rat TNF were shown to inhibit binding of ^{125}I-labeled human TNF to CrmE (Fig. 4). Similar findings were reported by Saraiva and Alcamí (2001) using soluble CrmE from CPV EP. The ability of CrmE to inhibit the cytotoxic activity of human TNF suggests a high affinity for human TNF and that this is sufficient to block interaction with cellular receptors.

Biological activity of membrane-bound VV USSR CrmE

In contrast to necrotic cell death, induction of apoptosis requires the activation of cysteine proteases of the caspase family and is accompanied by DNA fragmentation (Martelli *et al.,* 2001). Some poxviruses encode caspase inhibitors to prevent apoptotic cell death mediated by TNF or other inducers of apoptosis (Dobbelstein and Shenk, 1996; Kettle *et al.,* 1997). To investigate if expression of cell surface vTNFRs could be another mechanism by which VV-infected cells inhibit apoptotic cell death, TNF-mediated apoptotic cell death was assessed by cell cycle analysis using propidium iodide (PI) to stain the DNA of human Saos2 cells that had been treated with hTNF (5 U/ml) and CHX (10 μg/ml) following mock infection or infection with different viruses. Apoptotic cells were identified by subdiploid staining of DNA due to activation of endogenous endonuclease activity and subsequent leakage of low-molecular-weight DNA products. The VV WR gene B13R encodes an intracellular polypeptide that protects virus-infected cells from TNF-mediated apoptosis (Kettle *et al.,* 1997). As expected, cells infected with a deletion mutant lacking the B13R gene were more sensitive to hTNF-mediated apoptosis than those infected with wild-type virus or with a revertant virus in which the B13R gene had been reinserted into the B13R deletion mutant (Fig. 7). Immunoblot analysis using a rabbit antiserum raised to the WR B13R ORF expressed as a glutathione *S*-transferase–fusion protein identified a 38.5-kDa protein in WR- but not USSR-infected BS-C-1 cells (Kettle, 1995); however, despite the absence of the B13R counterpart, USSR-infected cells were more resistant to hTNF-mediated apoptosis than uninfected cells (Fig. 7). Cells infected with

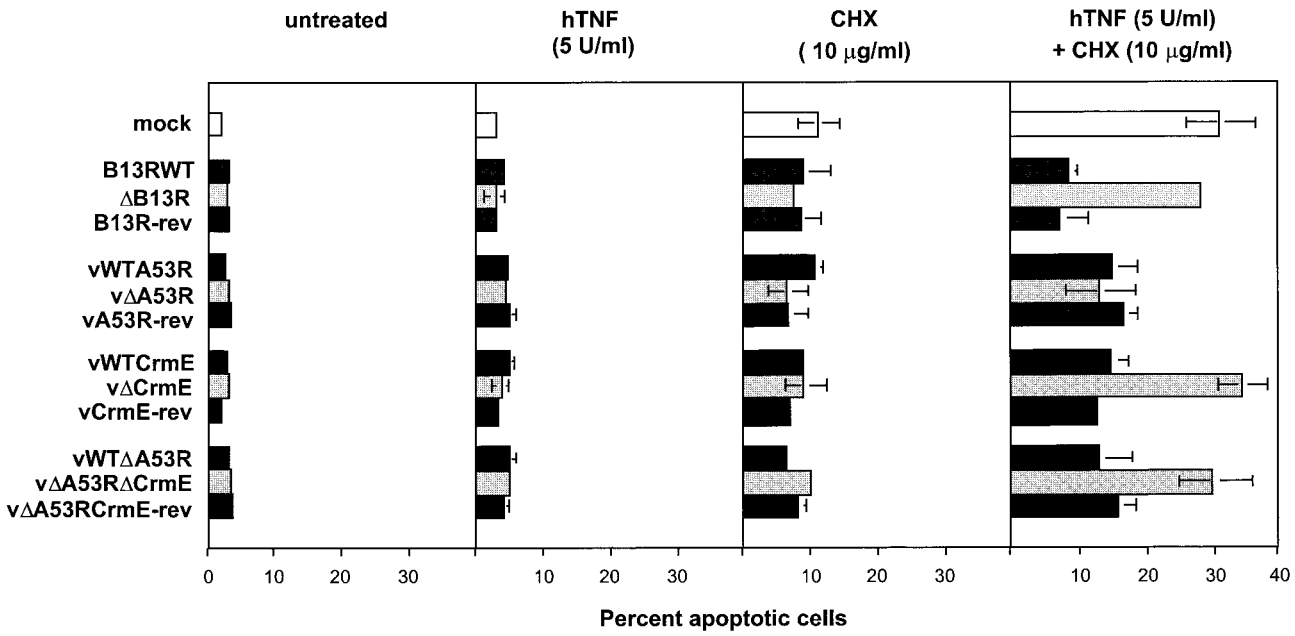

FIG. 7. Inhibition of TNF-mediated apoptosis by VV vTNFRs. Human Saos2 cells were mock-infected or infected with the indicated VVs for 6 h, washed, and incubated for an additional 6 h in the presence or absence of 10 μg/ml CHX. At 12 h p.i., hTNF was added and the cells were incubated for an additional 12 h. Apoptosis was assessed at 24 h p.i. by cell cycle analysis after incubation with PI as described under Materials and Methods.

deletion mutants lacking USSR CrmE (vΔCrmE or vΔA53RΔCrmE) were sensitive to TNF-mediated apoptosis while cells infected with vΔA53R remained resistant, suggesting a role for CrmE, but not A53R, in protection against hTNF-mediated apoptosis.

To determine if the protective effect of CrmE was due to expression of the cell surface vTNFR, cells were pretreated for 6 h with CHX, washed to remove any secreted CrmE, and incubated for a further 12 h with or without hTNF. After this time supernatants were removed and assessed for binding of ^{125}I-hTNF as a measure of soluble CrmE activity and infected cells were processed for PI staining. Pretreatment of USSR vΔA53R-infected cells with CHX reduced CrmE secretion by >85% (no CHX = 7654 ± 655 cpm bound, CHX treatment = 1061 ± 213 cpm bound) and addition of this CHX-treated supernatant to vΔA53RΔCrmE-infected cells failed to enhance their resistance to hTNF-mediated apoptosis (data not shown), indicating that the levels of soluble CrmE secreted in the presence of CHX are not sufficient to protect cells from TNF-mediated apoptosis. Together these findings suggest that expression of cell surface CrmE is sufficient to inhibit TNF-mediated apoptosis of cells infected with VV USSR.

The role of VV vTNFRs in virulence

To assess whether VV vTNFRs affect virus virulence, recombinant USSR viruses with deletions in A53R, in CrmE, or in both vTNFRs were examined in murine intradermal and intranasal models. Initial studies in the intradermal model showed that BALB/c mice infected with increasing doses of VV USSR (up to 10^6 PFU) failed to develop, or developed only very small lesions, when compared to mice infected with 10^4 PFU of VV WR (data not shown). Furthermore, deletion of A53R or VV CrmE failed to affect lesion size. Viruses were then compared in the intranasal model of infection. Mice were infected with 10^4 PFU of WR or with 10^6 PFU of USSR-derived recombinant viruses, and weight loss was assessed daily (Fig. 8). Mice infected with 10^4 PFU of VV WR lost weight rapidly and were sacrificed on day 7 p.i. when >25% of their original body weight had been lost (Fig. 8). In contrast, mice infected with a 100-fold higher dose of VV strain USSR showed only a mild loss and a rapid regain in weight with no visible signs of illness. Deletion of gene A53R from VV USSR had no effect on virulence for mice in this model (Fig. 8A); however, mice infected with either vΔCrmE (Fig. 8B) or vΔA53RΔCrmE (Fig. 8C) were attenuated markedly and lost little weight when compared to wild-type and revertant controls.

To assess further the role of VV vTNFRs *in vivo,* mice were infected intranasally with rVVs expressing Lister A53R (vA53RLister) or USSR CrmE (vCrmEUSSR). A rVV expressing an irrelevant protein from the TK locus, the hemagglutinin from influenza virus A/Jap/305/57 (vH2Influenza; Smith *et al.,* 1983), was also included. Compared to VV WR, vH2Influenza was attenuated markedly in the intranasal model (Fig. 9), consistent with previous reports that disruption of the TK gene reduces virulence in this model (Williamson *et al.,* 1990; Taylor *et al.,* 1991). However, rVVs expressing either Lister A53R or USSR CrmE displayed enhanced virulence compared to this TK$^-$ control. In particular, mice infected with vCrmEUSSR lost weight rapidly and were sacrificed 8–10

days p.i. (Fig. 9A). Signs of illness also increased rapidly after day 5 p.i. in vCrmEUSSR-infected animals (Fig. 8B), and all mice in this group showed symptoms of pneumonia at the time of sacrifice. Infection with vA53RLister was accompanied by a smaller weight loss (Fig. 9A) and a modest and transient increase in signs of illness (Fig. 9B), and infected mice recovered from the infection. Together, these results indicate that either loss (as seen with vΔCrmE or vΔA53RΔCrmE viruses) or overexpression (as seen with vCrmEUSSR or vA53RLister viruses) of VV vTNFRs can influence virus virulence in the intranasal model.

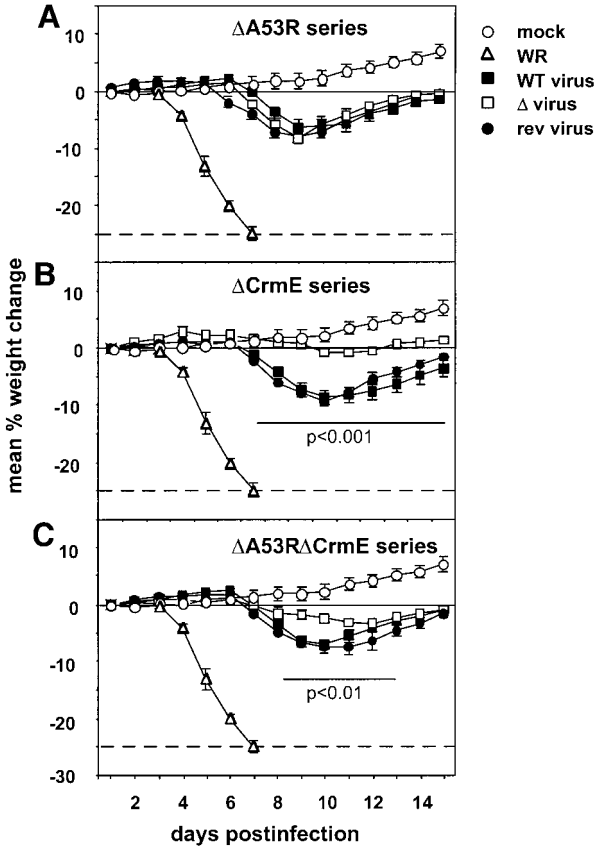

FIG. 8. Deletion of CrmE, but not A53R, attenuates VV USSR infection in mice. Groups of five mice were mock-infected (○) or infected with 10^4 PFU or WR (△) or 10^6 PFU of (A) A53R-deletion series of viruses vWTA53R (■), vΔA53R (□), or vA53R-rev (●); (B) CrmE-deletion series viruses vWTCrmE (■), vΔCrmE (□), or vCrmE-rev (●); (C) vWTΔA53R (■), vΔA53RΔCrmE (□), or vΔA53RCrmE-rev (●). Mice were weighed daily and results are expressed as the mean percentage weight loss of each group ± SEM, compared with the weight immediately prior to infection. *P*-values were determined using Student's *t* test and indicate mean percentage weight changes of mice infected with Δ viruses that were significantly different from those of mice infected with the corresponding WT viruses; (B) $P < 0.001$, days 6 to 14 and (C) $P < 0.02$; days 7 to 12. All mice infected with WR were sacrificed when they had lost >25% of their original body weight. No other animals were sacrificed. All animals were also monitored daily for signs of illness, scored from 1 to 4. None of the USSR-infected mice showed any signs of illness, while WR-infected mice were sacrificed at 8 days p.i., when all mice scored 3.

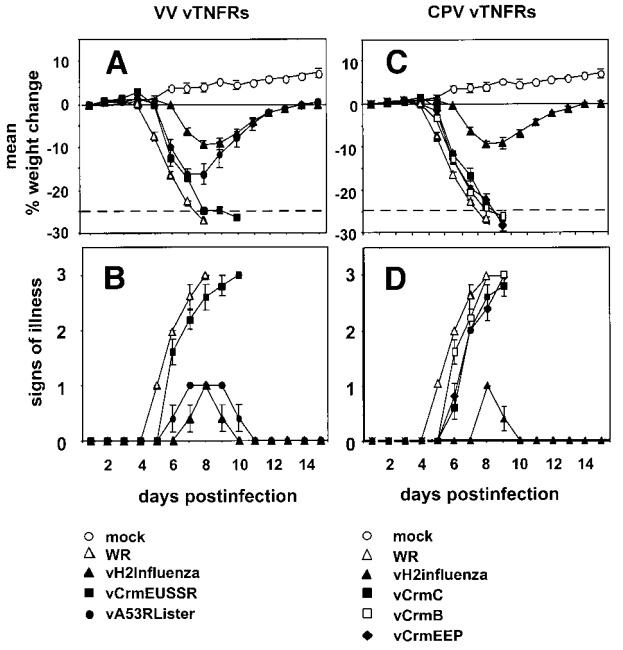

FIG. 9. Virulence of rVVs expressing vTNFRs from VV or CPV. Groups of five mice were mock-infected (○) or infected (A and B) with 10^4 PFU of WR (△) or 10^6 PFU of rVVs vH2Influenza (▲), vA53RLister (●), or vCrmE-USSR (■) or (C and D) with 10^4 PFU or WR (△) or 10^6 PFU of rVVs vH2Influenza (▲), vCPVCrmB (□), vCPVCrmC (■), or (◆). Mice vCrmEEP were weighed daily and results are expressed as the mean percentage weight loss of each group ± SEM, compared with the weight immediately prior to infection. The mean value of signs of illness ± SEM in each group are shown. All mice in groups infected with WR, vCrmE-USSR, vCPVCrmB, vCPVCrmC, or vCrmEEP were sacrificed when their mean percentage weight reached −25%. None of the vH2Influenza-, vA53RLister-, or PBS-inoculated mice were sacrificed.

Virulence of rVVs expressing CPV vTNFRs

A number of TNF-binding proteins have been characterized from strains of CPV; however, their contribution to virulence in an animal model has not been examined. We have examined the effect of CPV CrmB, CrmC, and CrmE on the virulence of TK^- VV WR in mice using rVVs, which express each of these proteins under the control of a strong synthetic promoter. Mice infected with vCrmB, vCrmC, or vCrmEEP lost weight rapidly and were sacrificed 8 to 9 days p.i., whereas a much milder disease was noted in mice infected with vH2Influenza (Figs. 9C and 9D).

DISCUSSION

In this study we have characterized the soluble and cell surface vTNFR activity encoded by VV strain USSR. As in VV Lister (Alcamí *et al.,* 1999), soluble vTNFR activity in VV USSR was encoded by the A53R gene, with the B28R gene being inactive due to fragmentation. Furthermore, we have identified an additional vTNFR in VV USSR, Lister, and Evans, closely related to CPV EP CrmE, which mediates both soluble and cell surface TNF-binding activity. Although secreted and membrane-bound VV

CrmE bound human, rat, and mouse TNF, only human TNF was inhibited in *in vitro* cytotoxicity assays. Finally, we have examined the role of VV vTNFRs *in vivo* and report that deletion of VV CrmE, but not A53R, from VV USSR led to a marked attenuation of the virus in the murine intranasal model of infection.

All vTNFRs identified in poxviruses show sequence similarity to the N-terminal ligand-binding domains of cellular TNFRs, but lack transmembrane anchoring and cytoplasmic signaling domains. VV CrmE has these characteristics but unlike other vTNFRs also has membrane-bound vTNFR activity. Cell surface vTNFR was not due to soluble vTNFR binding back to the cell surface, because supernatants from USSR-infected cells did not confer cell surface TNF-binding activity to mock-infected or vΔCrmE-infected cells, nor is this activity dependent upon interaction with another VV-encoded protein since activity was expressed at the surface of insect cells infected with recombinant baculoviruses expressing CrmE. Perhaps CrmE is transported to the cell surface via interactions with cellular proteins, where it is retained for a period of time before detachment or cleavage from the cell surface. Alternatively, the N-terminal signal sequence may function as an anchor, such that CrmE is retained at the cell surface with a type II membrane topology, leaving the CRDs exposed to bind soluble TNF. In this conformation some CrmE might be released after proteolysis.

In a previous report, cells infected with CPV strain EP or with recombinant VV expressing EP CrmE were found to express soluble, but not membrane-bound, TNF-binding activity (Saraiva and Alcamí, 2001), which is surprising given its very close sequence similarity to the VV CrmE protein. However, we have found that EP-infected TK^-143, BS-C-1, and RK_{13} cells express soluble and cell surface vTNFRs (data not shown). Expression of both soluble and cell surface vTNFR activity tends to be somewhat lower when compared with VV USSR, but is enhanced by increasing the m.o.i. to 20 PFU/cell or by incubating virus-infected cells an additional day prior to assay. Furthermore, TK^-143B cells or insect cells infected with rVV or recombinant baculovirus expressing EP CrmE were found to express soluble and membrane-bound vTNFR activity (data not shown). It would be of interest to determine if CPV strain GRI-90 encodes both soluble and cell surface vTNFR activity, given that it also differs from VV CrmE by only a single amino acid.

The cell surface vTNFR encoded by VV strains USSR, Lister, and Evans is interesting in that the majority of cytokine receptors or binding proteins described in poxviruses to date are secreted. An exception is the B18R protein that is secreted from WR-infected cells and can bind to infected and uninfected cells and protect these against the induction of an antiviral state by IFN-α/β, thereby maintaining the cells' susceptibility to virus infections (Alcamí *et al.,* 2000). In contrast, CrmE is present at the surface of virus-infected, but not uninfected, cells, where it may act to protect these cells from TNF-mediated cell killing. Virus-infected cells are killed selectively by TNF and this activity is accelerated by IFN-γ (Mestan *et al.,* 1986; Wong and Goeddel, 1986; Wong *et al.,* 1988); thus CrmE expressed at the cell surface could bind TNF, protect the cell from TNF-mediated killing, and promote the synthesis and release of viral progeny. Secreted CrmE binds soluble TNF and can inhibit its interaction with cellular receptors and may thereby hinder the proinflammatory and antiviral properties of this cytokine. Thus, secreted and cell surface vTNFRs could play distinct but complementary roles in manipulating the host response to infection.

It is curious that most VV strains do not express vTNFR activity, yet USSR, Lister, and Evans encode two TNF binding proteins. Other orthopoxviruses express multiple vTNFRs simultaneously, suggesting that they may modulate the immune system in different ways. CPV strain Brighton Red encodes three soluble TNFRs, CrmB, CrmC, and CrmD (Hu *et al.,* 1994; Smith *et al.,* 1996; Loparev *et al.,* 1998), while an additional vTNFR, CrmE, has been reported in CPV strain EP (Saraiva and Alcamí, 2001). CPV strain GRI-90 sequence data reveal five distinct ORFs with sequence similarity to TNFRs (Shchelkunov *et al.,* 1998). Counterparts of CrmB (B28R/C22L), CrmC (A53R), and now CrmE have been identified and characterized in VV, although only the latter two encode functional vTNFRs. It is unlikely that VV USSR expresses additional vTNFRs, since in numerous assays we have shown that deletion of both A53R and CrmE removes virtually all soluble and cell surface TNF-binding activity (Fig. 2; and data not shown). Combinations of specific oligonucleotide primers failed to identify a CrmD counterpart in genomic DNA from any of the 15 VV strains tested in this study (data not shown). In addition, probes generated from PCR-derived CrmD DNA failed to hybridize to USSR, Lister, or Evans genomic DNA in Southern blot analysis (data not shown), indicating that expression of a CrmD counterpart is unlikely.

We have examined the *in vivo* role of VV vTNFRs in murine intradermal and intranasal models, thereby examining the effect of loss of VV vTNFRs in the context of local and systemic infections, respectively. Studies in the intradermal model were complicated by the poor ability of wild-type USSR to induce lesions in the ears of infected mice; however, deletion of vTNFRs did not alter lesion size significantly. USSR was slightly more virulent in the murine intranasal model; lung virus titers were low, there was no detectable viremia, and no virus was recovered from other organs such as brain and spleen (unpublished observations). This mild infection is attenuated further by the loss of the CrmE vTNFR, demonstrating that vTNFRs may act as virulence factors in VV infection. Expression of VV (CrmC and CrmE) or CPV (CrmB, CrmC, and CrmE) vTNFRs from TK^- rVVs enhanced virulence in the murine intranasal model, further demonstrating the ability of poxvirus-encoded TNF-bind-

ing proteins to manipulate the host response to infection. Other studies have examined the role of poxvirus vTNFRs in the context of an *in vivo* infection. MT-2, a vTNFR encoded by myxoma virus, contributes to virulence in European rabbits (Upton *et al.,* 1991). In addition, CPV Brighton Red mutants lacking CrmB or CrmC have been examined in the chicken embryo chorioallantoic membrane model (Smith *et al.,* 1996), and the CrmB deletion virus was shown to have a higher LD_{50} than wild-type CPV in a mouse intracranial model of infection (Palumbo *et al.,* 1994). In our study the use of revertant viruses, whereby the vTNFR gene of interest (A53R or CrmE) is reinserted into the appropriate locus of the deletion virus, strengthens the conclusion that CrmE is a VV-encoded virulence factor in the murine intranasal model.

Deletion of CrmE, but not A53R, attenuated VV in the murine intranasal model despite the ability of A53R, but not USSR CrmE, to inhibit the cytotoxic activity of murine TNF for L929 cells. One explanation would be that levels of A53R vTNFR produced following infection of mice are low compared to VV CrmE and may not be sufficient to alter viral virulence. In initial studies we observed that deletion of A53R had little effect upon overall expression of soluble vTNFR activity in VV USSR (Fig. 1). In addition, the majority of soluble TNF-binding activity was lost following deletion of CrmE (vΔCrmE), despite the presence of an intact A53R (Fig. 2). Alcamí *et al.* (1999) reported that expression of Lister A53R from VV WR under a strong promoter did not produce as much vTNFR activity as that present in VV Lister supernatant although much higher levels of A53R protein were secreted into supernatants of cells infected with the recombinant virus (Alcamí *et al.,* 1999). Together, these findings indicate that the majority of soluble vTNFR activity expressed in VV strains USSR and Lister is encoded by CrmE. An alternative explanation could be that expression of membrane-bound CrmE may be particularly important in promoting virus dissemination and spread by protecting infected cells from TNF-mediated killing. Another possibility is that CrmE binds and inhibits another member of the TNF ligand superfamily that is important in the context of VV infection. In this regard, EP CrmE does not bind human TRAIL, GITR, CD40L, BAFF, TWEAK, 4-1BBL, or RANK (Saraiva and Alcamí, 2001).

VV strains USSR, Lister, and Evans are the only VV strains identified that secrete a CrmC-like vTNFR or produce soluble and cell surface vTNFRs via the production of CrmE. These strains share other common features including the expression of a chemokine-binding protein (Alcamí *et al.,* 1998) and a mild virulence for mice. Genomic sequencing upstream and downstream from the USSR CrmE gene indicated similarities with the sequence from CPV strain GRI-90 K3R(CrmE) flanking regions (data not shown). Furthermore, oligonucleotide primers designed to these flanking genes amplified products from genomic DNA extracted from VV USSR, Lister, and Evans, but not strains WR or Copenhagen. These results suggest that USSR, Lister, and Evans are closely related strains of VV and, in this region of the genome, are more closely related to CPV than other strains of VV.

In summary, our data demonstrate that VV USSR produces two distinct TNF-binding proteins: a soluble vTNFR encoded by the A53R and a CrmE-like protein that mediates both soluble and cell surface vTNFR activity.

MATERIALS AND METHODS

Cells and viruses

The orthopoxvirus strains used in this study and the growth conditions for African green monkey BS-C-1 and human TK^-143B and U937 cells have been described elsewhere (Alcamí and Smith, 1995; Alcamí *et al.,* 1998). Saos2, a human osteosarcoma cell line, was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The WR recombinant VVs expressing VV Lister A53R, CPV A53R/CrmC, and CPV B28R/CrmB have been described previously (Alcamí *et al.,* 1999). Working stocks of rVVs were prepared by sedimentation of cytoplasmic extracts of infected cells through a cushion of 36% (wt/vol) sucrose by centrifugation at 24,000 *g* for 80 min at 4°C.

Reagents

Radioiodinated human recombinant TNF (approximately 50 μCi/μg) was from DuPont–New England Nuclear. Recombinant human, murine, and rat TNF and human LTα were obtained from Peprotech, and each had a specific activity of $\geq 2 \times 10^7$ units/mg.

Plasmid constructions

VV strain USSR genomic DNA was used for cloning the A53R and B28R genes into pBluescript SK vector as follows. Gene A53R was located in an ~2.0-kb *Eco*RV fragment by Southern blot analysis of USSR virus DNA and hybridization with an A53R probe amplified by PCR. Following *Eco*RV digestion of the virus DNA, an ~2.0 kb fragment was cloned into *Eco*RV-pBS SK, and this plasmid was called pAK1. Similarly, the USSR B28R gene was located in an ~2.1 kb *Ava*I fragment in USSR virus DNA. The fragment was made blunt-ended by treatment with Klenow DNA polymerase and cloned into *Eco*RV-digested pBS, and the plasmid was named pAK3. Virus DNA cloned into plasmids pAK1 and pAK3 was sequenced using M13 forward and reverse universal primers and oligonucleotides specific for genes A53R (from WR) and B28R (Copenhagen/WR). Sequencing reactions were carried out using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and run on an ABI 373 DNA sequencer according to the manufacturer's instructions. Sequence data were analyzed using the Staden (Bonfield *et al.,* 1995) and GCG (Genetics Com-

puter Group) sequence assembly and analysis packages.

Plasmids were constructed for making VV USSR deletion mutants lacking A53R (pΔA53R) or VV CrmE (pΔCrmE) as follows. Oligonucleotides that flanked the left or right side of the A53R gene or the CrmE gene were used to generate PCR products that contained terminal restriction enzyme sites. For pΔA53R, 5′-CCCGTCGACTGCATGCGTGGTAGATG-3′ and 5′-CATAAGCTTATATTATCATATACTACAAT-3′ containing 5′ *Sal*I and *Hin*dIII sites (underlined), respectively, were used to amplify the left flank, and 5′-GGTAAGCTTTGCGACTACCT-3′ and 5′-CCCGGATCCAGCGTATATATGTAGAAATCG-3′ containing 5′ *Hin*dIII and *Bam*HI sites, respectively, were used to amplify the right flank. For pΔCrmE, 5′ TAAGGAATTCGGAAAGACATTATTACACG-3′ and 5′-ATTCGAGCTCGACACCTTGTTCACATTTC-3′ containing 5′ *Eco*RI and *Sac*I sites, respectively, were used to amplify the left flank, and 5′-TAAGGAGCTCTAAGACTTACTCGCATCTAC-3′ and 5′-ATTCGGATCCGCATAACGACATGCACATC-3′ containing *Sac*I and *Bam*HI sites, respectively, were used to amplify the right flank. The amplified products were digested with the relevant restriction enzymes and ligated into plasmid pSJH7 (Hughes *et al.,* 1991). Plasmid pΔA53R lacked 88% of the A53R ORF (codons 1–163) and pΔCrmE lacked 84% of the CrmE ORF (codons 27–167) and both plasmids contained the *Escherichia coli* guanine phosphoribosyl transferase (*Ecogpt*) gene (Boyle and Coupar, 1988) under the control of the VV p7.5K promoter (Mackett *et al.,* 1982). Plasmid pA53R-rev was constructed by generating a wild-type copy of the VV USSR A53R and flanking sequences by PCR using virus genomic DNA as template and cloning the products between the *Sal*I and the *Bam*HI sites of plasmid pSJH7. Plasmid pCrmE-rev was constructed in a similar manner by cloning VV USSR CrmE and flanking sequences between the *Eco*RI and the *Bam*HI sites of pSJH7. The sequence of PCR-derived inserts from all plasmids was confirmed by sequencing.

Construction of VV USSR deletion mutants

VV USSR deletion mutants were constructed by transient dominant selection using the *Ecogpt* gene as a selectable marker as described previously (Falkner and Moss, 1990). Briefly, CV-1 cells were infected with VV USSR and transfected with pΔA53R or pΔCrmE, and recombinant viruses were selected in the presence of mycophenolic acid (MPA). An intermediate plaque containing the full-length gene and its deleted version were resolved by plaque purification on the hypoxanthine guanine phosphoribosyl transferase (hgprt)-negative HeLa cell line D98OR in the presence of 6-thioguanine to form deletion (Δ) and wild-type (WT) plaque-purified viruses (vWTA53R and vΔA53R, and vWTCrmE and vΔCrmE). To construct a deletion virus lacking both A53R and CrmE, CV-1 cells were infected with VV vΔA53R and transfected with pΔCrmE, and MPA-resistant viruses were resolved into deletion (vΔA53RΔCrmE) or WT (vΔA53RWT) viruses. Revertant viruses (vA53R-rev, vCrmE-rev, and vΔA53RCrmE-rev) were constructed in a similar manner, using vΔA53R, vΔCrmE, or vΔA53RΔCrmE, respectively, as parent viruses and plasmids pA53R-rev (for vA53R-rev) and pCrmE-rev (for vCrmE-rev and vA53RCrmE-rev). All virus isolates were plaque-purified three times and their genomic structures were confirmed by PCR with primers flanking the relevant gene (A53R, CrmE, or both) and with primers corresponding to the appropriate flanking genes, and by Southern blot analysis using DNA extracted from virus cores (Esposito *et al.,* 1981) and a probe corresponding to the region deleted in the A53R or CrmE genes.

Construction of recombinant baculoviruses

VV USSR CrmE was amplified by PCR using oligonucleotides 5′-ATTGGGATCCCATTATTGACTTAACGATGACG-3′ and 5′-ATTGCTCGAGTTATCTTGTCATTGGTTTACATTG-3′ that introduced *Bam*HI and *Xho*I sites (underlined), respectively. The DNA fragment was digested with these enzymes and cloned into *Bam*HI and *Xho*I-cut pBAC-1 (R&D Systems, Abingdon, U.K.) generating plasmid pAcCrmE so that the CrmE ORF was downstream of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedron gene promoter. Plasmid pAcCrmE and linear AcNPV DNA (BacPAK6, Clontech) were used to construct recombinant baculoviruses AcCrmEUSSR by transfection into Sf21 cells (Alcamí and Smith, 1995).

Construction of rVVs

VV USSR gene CrmE was amplified by PCR with virus DNA as template and oligonucleotides 5′-ATTGGGATCCCATTATTGACTAAACGATGACG-3′ and 5′-TAACCCGCGGCTTATCTTGTCATTGGTTTAC-3′ that contained *Bam*HI and *Sac*II sites (underlined), respectively. Resultant products were cloned into *Bam*HI and *Sac*II-digested pMJ601 (Davison and Moss, 1990) to create plasmid pTKCrmE and the sequence of the insert was confirmed by DNA sequencing. The plasmid was transfected into VV WR-infected cells and TK$^-$ rVVs were screened for β-galactosidase expression and plaque-purified three times (Chakrabarti *et al.,* 1985). The presence of VV CrmE in the rVV (designated vCrmEUSSR) was confirmed by PCR analysis. A similar strategy, using CPV EP DNA as a template, was employed to generate a TK$^-$ rVV expressing the CrmE gene from EP (vCrmEEP).

Metabolic labeling of proteins and electrophoretic analysis

Sf21 cells or TK$^-$143B cells were infected with recombinant baculoviruses or VVs, respectively, at 10 PFU/cell. At the indicated times postinfection, cultures were washed and labeled for 2 h with 70 μCi of Pro-mix L-[^{35}S]methionine and L-[^{35}S]cysteine *in vitro* cell labeling mix (>1000 Ci/mmol, Amersham) in methionine- and cysteine-free medium in the absence of serum. Super-

natants were harvested and clarified by centrifugation, and cells were dissociated in sample buffer. Supernatants and cells were analyzed by SDS–PAGE in 15% gels and by fluorography with Amplify (Amersham).

Cytolytic assays

The ability of VV vTNFRs to inhibit TNF or LTα cytotoxicity for L929 cells was examined using a crystal violet staining method (Smith *et al.,* 1996). Briefly, serial dilutions of TNF or LTα were incubated at 4°C for 1 h with supernatants from TK$^-$143B cells that were mock-infected or infected for 24 h at 10 PFU/cell with WR, vA53RLister, or vCrmEUSSR. Samples were incubated with 5×10^4 L929 cells/well in 96-well plates in the presence of 1 μg/ml actinomycin D at 37°C for 12 h and stained with 0.5% crystal violet in methanol/water (1/4). All assays were performed in triplicate and the percentage cytotoxicity was calculated as $(OD_{medium} - OD_{TNF})/OD_{medium}$.

Apoptosis assays

Apoptosis was assessed by cell cycle analysis using PI staining and flow cytometry (Vermes *et al.,* 2000). Human Saos2 cells were grown to confluency in 6-well plates and infected with VV at 5 PFU/cell for 6 h. Cells were washed with DMEM and left untreated or treated with 10 μg/ml cycloheximide (CHX). After 6 h, the cells were washed in DMEM and treated with TNFα, CHX, or TNF/CHX for 10 h at 37°C. The cells were detached in PBS containing 4 mM EDTA, washed twice, and fixed in 80% methanol at 4°C for 4 h before being stained with 50 μg/ml PI and 50 μg/ml of RNase A. At least 10,000 stained cells were examined for DNA content by flow cytometry and apoptotic cells were located in the subdiploid region (i.e., the cell population showing increased DNA fragmentation). A marker was set on the subdiploid region in uninfected and untreated cells, and this marker was applied to all other cell populations tested. Cell debris was excluded from analysis using forward scatter/side scatter gating.

Cell surface and soluble TNF-binding assays

Supernatants from VV-infected TK$^-$143B cells or baculovirus-infected Sf21 cells were harvested at 1 or 3 days p.i., respectively, and prepared as described (Alcamí and Smith, 1992). All binding and competition assays were carried out in duplicate using RPMI medium containing 1% FBS and 20 mM HEPES, pH 7.5. Soluble TNF-binding assays were performed by incubating supernatants, equivalent to $\sim 10^5$ cells, with 100–250 pM human recombinant ^{125}I-TNF in a final volume of 150 μl for 2 h at room temperature. The ligand–receptor complexes were precipitated with polyethylene glycol (PEG) and the precipitate was collected on Whatman GF/C filters as described (Alcamí and Smith, 1992). Nonspecific binding precipitated with binding medium alone or determined in the presence of excess unlabeled TNF was subtracted. In the cell surface-binding assay, uninfected or infected TK$^-$143B cells were detached from plates by incubation with 10 mM EDTA in phosphate-buffered saline. Cells (1.5×10^6) were washed twice with binding medium (1% FBS, 20 mM HEPES, RPMI medium) by centrifugation and incubated in binding medium with 100–250 pM human recombinant ^{125}I-TNF for 2 h at 4°C in 150 μl. Bound ^{125}I-TNF was determined by phthalate oil centrifugation as described (Alcamí and Smith, 1992).

Virulence assays in mice

For intradermal infections, groups of five female BALB/c mice (6 weeks old) were anesthetized and either 10^4 or 10^6 PFU of VV in PBS was injected intradermally into the left ear pinna. The diameter of lesions was estimated daily to the nearest 0.5 mm using a micrometer. For intranasal infections, groups of five female BALB/c mice (6 weeks old) were inoculated under general anesthesia with viruses in 20 μl of PBS. Each day, mice were weighed individually and monitored for signs of illness, and those suffering a severe infection or having lost 25% of their original body weight were sacrificed.

ACKNOWLEDGMENTS

This work was supported by Wellcome Trust Programme Grant 037575. P.R. is Howard Florey Fellow and G.L.S. is a Wellcome Trust Principal Research Fellow.

REFERENCES

Aguado, B., Selmes, I. P., and Smith, G. L. (1992). Nucleotide sequence of 21.8 kbp of variola major virus strain Harvey and comparison with vaccinia virus. *J. Gen. Virol.* **73,** 2887–2902.

Alcamí, A., Khanna, A., Paul, N. L., and Smith, G. L. (1999). Vaccinia virus strains Lister, USSR and Evans express soluble and cell surface tumor necrosis factor receptor. *J. Gen. Virol.* **80,** 949–959.

Alcamí, A., and Koszinowski, U. H. (2000). Viral mechanisms of immune evasion. *Trends Microbiol.* **8,** 410–418.

Alcamí, A., and Smith, G. L. (1992). A soluble receptor for interleukin-1 beta encoded by vaccinia virus: A novel mechanism of virus modulation of the host response to infection. *Cell* **71,** 153–167.

Alcamí, A., and Smith, G. L. (1995). Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. *J. Virol.* **69,** 4633–4639.

Alcamí, A., Symons, J. A., Collins, P. D., Williams, T. J., and Smith, G. L. (1998). Blockade of chemokine activity by a soluble chemokine binding protein from vaccinia virus. *J. Immunol.* **160,** 624–633.

Alcamí, A., Symons, J. A., and Smith, G. L. (2000). The vaccinia soluble IFN-α/β receptor binds to the cell surface and protects cells from the anti-viral effects of IFN. *J. Virol.* **74,** 11230–11239.

Bonfield, J. K., Smith, K., and Staden, R. (1995). A new DNA sequence assembly program. *Nucleic Acids Res.* **23,** 4992–4999.

Boyle, D. B., and Coupar, B. E. H. (1988). A dominant selectable marker for the construction of recombinant poxviruses. *Gene* **65,** 123–128.

Chakrabarti, S., Brechling, K., and Moss, B. (1985). Vaccinia virus expression vector: Coexpression of β-galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5,** 3403–3409.

Davison, A. J., and Moss, B. (1990). New vaccinia virus recombination plasmids incorporating a synthetic late promoter for high level expression of foreign proteins. *Nucleic Acids Res.* **18,** 4285–4286.

Dobbelstein, M., and Shenk, T. (1996). Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. *J. Virol.* **70,** 6479–6485.

Esposito, J. R., Condit, R. C., and Obijeski, J. (1981). The preparation of orthopoxvirus DNA. *J. Virol. Methods* **2,** 175–179.

Falkner, F. G., and Moss, B. (1990). Transient dominant selection of recombinant vaccinia viruses. *J. Virol.* **64,** 3108–3111.

Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179,** 247–266.

Goossens, V., Grooten, J., De Vos, K., and Fiers, W. (1995). Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl. Acad. Sci. USA* **92,** 8115–8119.

Howard, S. T., Chan, Y. S., and Smith, G. L. (1991). Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology* **180,** 633–647.

Hu, F. Q., Smith, C. A., and Pickup, D. J. (1994). Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. *Virology* **204,** 343–356.

Hughes, S. J., Johnston, L. H., de Carlos, A., and Smith, G. L. (1991). Vaccinia virus encodes an active thymidylate kinase that complements a cdc8 mutant of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266,** 20103–20109.

Kettle, S. (1995). Characterisation of vaccina virus gene B13R. D.Phil. University of Oxford, Oxford.

Kettle, S., Alcamí, A., Khanna, A., Ehret, R., Jassoy, C., and Smith, G. L. (1997). Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. *J. Gen. Virol.* **78,** 677–685.

Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: Integrating mammalian biology. *Cell* **104,** 487–501.

Loparev, V. N., Parsons, J. M., Knight, J. C., Panus, J. F., Ray, C. A., Buller, R. M., Pickup, D. J., and Esposito, J. J. (1998). A third distinct tumor necrosis factor receptor of orthopoxviruses. *Proc. Natl. Acad. Sci. USA* **95,** 3786–3791.

Lucin, P., Jonjic, S., Messerle, M., Polic, B., Hengel, H., and Koszinowski, U. H. (1994). Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. *J. Gen. Virol.* **75,** 101–110.

Mackett, M., Smith, G. L., and Moss, B. (1982). Vaccinia virus: A selectable eukaryotic cloning and expression vector. *Proc. Natl. Acad. Sci. USA* **79,** 7415–7419.

Martelli, A. M., Zweyer, M., Ochs, R. L., Tazzari, P. L., Tabellini, G., Narducci, P., and Bortul, R. (2001). Nuclear apoptotic changes: An overview. *J. Cell. Biochem.* **82,** 634–646.

Massung, R. F., Liu, L. I., Qi, J., Knight, J. C., Yuran, T. E., Kerlavage, A. R., Parsons, J. M., Venter, J. C., and Esposito, J. J. (1994). Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975. *Virology* **201,** 215–240.

Mestan, J., Digel, W., Mittnacht, S., Hillen, H., Blohm, D., Moller, A., Jacobsen, H., and Kirchner, H. (1986). Antiviral effects of recombinant tumour necrosis factor in vitro. *Nature* **323,** 816–819.

Ng, A., Tscharke, D. C., Reading, P. C., and Smith, G. L. (2001). The vaccinia virus A41L protein is a soluble 30 kDa glycoprotein that affects virus virulence. *J. Gen. Virol.* **82,** 2095–2105.

Palumbo, G. J., Buller, R. M. L., and Glasgow, W. C. (1994). Multigenic evasion of inflammation by poxviruses. *J. Virol.* **68,** 1737–1749.

Sambhi, S. K., Kohonen-Corish, M. R., and Ramshaw, I. A. (1991). Local production of tumor necrosis factor encoded by recombinant vaccinia virus is effective in controlling viral replication in vivo. *Proc. Natl. Acad. Sci. USA* **88,** 4025–4029.

Saraiva, M., and Alcamí, A. (2001). CrmE, a novel soluble tumor necrosis factor receptor encoded by poxviruses. *J. Virol.* **75,** 226–233.

Shchelkunov, S. N., Safronov, P. F., Totmenin, A. V., Petrov, N. A., Ryazankina, O. I., Gutorov, V. V., and Kotwal, G. J. (1998). The genomic sequence analysis of the left and right species-specific terminal region of a cowpox virus strain reveals unique sequences and a cluster of intact ORFs for immunomodulatory and host range proteins. *Virology* **243,** 432–460.

Shchelkunov, S. N., Totmenin, A. V., Loparev, V. N., Safronov, P. F., Gutorov, V. V., Chizhikov, V. E., Knight, J. C., Parsons, J. M., Massung, R. F., and Esposito, J. J. (2000). Alastrim smallpox variola minor virus genome DNA sequences. *Virology* **266,** 361–386.

Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* **248,** 1019–1023.

Smith, C. A., Hu, F. Q., Smith, T. D., Richards, C. L., Smolak, P., Goodwin, R. G., and Pickup, D. J. (1996). Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LT alpha. *Virology* **223,** 132–147.

Smith, G. L. (2000). Secreted poxvirus proteins that interact with the immune system. *In* "Effects of Microbes on the Immune System" (M. W. Cunningham and R. S. Fujinami, Eds.), pp. 491–507. Lippincott Williams & Wilkins, Philadelphia.

Smith, G. L., Murphy, B. R., and Moss, B. (1983). Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc. Natl. Acad. Sci. USA* **80,** 7155–7159.

Taylor, G., Stott, E. J., Wertz, G., and Ball, A. (1991). Comparison of the virulence of wild-type thymidine kinase (tk)-deficient and tk+ phenotypes of vaccinia virus recombinants after intranasal inoculation of mice. *J. Gen. Virol.* **72,** 125–130.

Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology* **184,** 370–382.

Vermes, I., Haanen, C., and Reutelingsperger, C. (2000). Flow cytometry of apoptotic cell death. *J. Immunol. Methods* **243,** 167–190.

Williamson, J. D., Reith, R. W., Jeffrey, L. J., Arrand, J. R., and Mackett, M. (1990). Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route. *J. Gen. Virol.* **71,** 2761–2767.

Wong, G. H., and Goeddel, D. V. (1986). Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. *Nature* **323,** 819–822.

Wong, G. H., Krowka, J. F., Stites, D. P., and Goeddel, D. V. (1988). In vitro anti-human immunodeficiency virus activities of tumor necrosis factor-alpha and interferon-gamma. *J. Immunol.* **140,** 120–124.